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(54) Method for detecting target nucleic acid.

(57) A method for detecting a target nucleic acid comprises the steps of reacting a sample with a probe in the presence of two or more kinds of reagents capable of being made an irreversible change capable of being detected and accumulating by an interaction through a double helix structure under a condition enabling the replication of the formation and dissociation of a hybrid composed of the target nucleic acid in the sample and the probe, accumulating the irreversible change caused by the interaction of the reagents, and then detecting the accumulated change.

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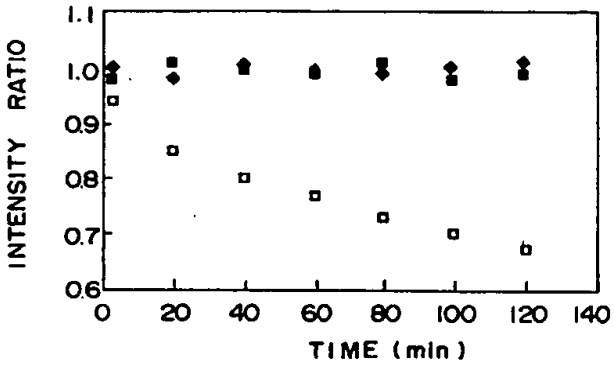

INTENSITY RATIO
1.1
1.0
0.9
0.8
0.7
0.6
0
20
40
60
80
100
120
140
TIME (min)

FIG. 1

BACKGROUND OF THE INVENTION

Field of the Invention

The present invention relates to a method for detecting or identifying a desired base sequence of a nucleic acid (DNA or RNA) of a virus, a microorganism, an animal, a plant or a man, or a method for detecting presence or absence of a variant in the base sequence, and a probe for use in the method.

Related Background Art

Many varied genes have been found owing to the development of the analytical technique of nucleic acids, and various kinds of hereditary diseases based on the variation of genes are being elucidated. It is now apparent that in such hereditary diseases, the bases of the gene are partially lacked, or the point mutation of the bases occurs, so that protein varies and various symptoms appear. At present, these hereditary diseases are mainly found by an assay using an enzyme or an immune technique using an antibody after the symptom appears. However, from the viewpoint of early treatment, it is important to early found the presence of the variant on the gene before the serious symptom appears.

As techniques for detecting the change of DNA or RNA of such a varied gene, there are usually an RFLP (restriction fragment length polymorphism) method and a method for determining the base sequence of DNA. However, when such a disease as mentioned above is diagnosed, several ten to several thousand copies of DNA or RNA are only obtained on occasion, and in the RFLP and the base sequence determination method, such a high sensitivity as to detect a small number of the DNA or RNA copies cannot be expected.

In recent years, there have been suggested some methods by which DNA or RNA can be detected even from a small number of its copies, but they have not been put to practical use because of a low S/N ratio or a poor reliability. Therefore, at present, when DNA or RNA is detected on the basis of a small number of its copies, it is a usual manner to increase, i.e., amplify the target DNA or RNA.

As a means for amplifying DNA or RNA, a PCR (polymerase chain reaction) method is now widely utilized. For example, the amplification of DNA by the PCR method is carried out as follows:

(1) Two kinds of oligonucleotide primers of about 20-mer are prepared. These primers are complementary to the 5' side of each chain of a target double-strand DNA having about 200 base pairs and containing a base sequence to be detected therein.

(2) In about 100 μl of a suitable buffer solution are dissolved the target DNA, the above-mentioned two kinds of oligonucleotide primers (100 pmol, respectively) and deoxynucleotide triphosphoric acids (1.25 mM, respectively) of four kinds of bases (adenine, guanine, cytosine and thymine).

(3) The solution is heated at 94 • C for 5 minutes to denature the target double-strand DNA.

(4) 10 units of a heat-resistant DNA polymerase (e.g., Taq NDA polymerase) are added to the solution.

(5) Annealing is done at 50 • C for 2 minutes.

(6) A polymerization reaction is carried out at 72 • C for 3 minutes.

(7) The above-mentioned steps (3) to (6) [except the step (4)] are repeated as many as required (25 to 30 cycles).

(8) The amplified DNA is extracted by a suitable treatment.

The thus amplified DNA is detected and analyzed by the above-mentioned RFLP, base sequence determination method, or the like.

As described above, according to the PCR method, such a trace amount of a nucleic acid as is not detected by a usual detection technique is amplified, whereby the detection is made possible. However, this method also has the following problems.

(1) The amplification and the detection are carried out by the utterly different means, and so a serial operation of the amplification, the extraction of nucleic acid and the detection is required until the final detection, which takes much labor and much time.

(2) The proceed of the polymerization depends upon the base sequence, the length and the like of the primer, and in a certain case, the polymerization does not proceed at all or a polymerization rate is extremely low. Furthermore, the unnecessary amplification is carried out owing the miss-priming of the primer. The reasons for these phenomenons are not definitely understood, but it can be supposed that they are concerned with the fact that the polymerization reaction is made with the aid of an enzyme. Therefore, in order to select the primer, many experiments with trial and error are often required.

(3) A temperature cycle is used for the amplification, and thus, in order to change a temperature difference of 40 • C or more at a high velocity, a device capable of programming the temperature with

respect to time is necessary. In the concrete, a heater and a cooler having a large capacity are necessary, which leads to the increase of cost.

(4) In compliance with the demand of a detecting system side, the nucleic acid portion of 200 or more bases is amplified, but in fact, PCR cannot amplify the nucleic acid portion of 20 to 30 bases in principle. However, a certain disease can be specified sufficiently by recognizing the sequence of about 20 bases in a certain case. If the PCR method is used for such a case, the nucleic acid portion is excessively amplified, which leads to the loss of time and the waste of expensive reagents.

SUMMARY OF THE INVENTION

In view of the above-mentioned problems of conventional techniques, the present invention has been intended, and an object of the present invention is to provide a method for detecting a target nucleic acid. According to the detecting method of the present invention, a small amount of a sample can be detected efficiently in one system under easily controllable reaction conditions without beforehand carrying out the amplification means of the target nucleic acid, and thus the detecting method of the present invention is suitable for the analysis of a trace amount of the sample.

Another object of the present invention is to provide a method for detecting a target nucleic acid which permits analysis at a low cost by the use of a simply constituted apparatus.

The above-mentioned objects can be achieved by the present invention.

That is, the present invention is directed to a method for detecting a target nucleic acid which comprises the steps of reacting a sample with a probe in the presence of two or more reagents capable of being made an irreversible change capable of being detected and accumulating by an interaction through a double helix structure under a condition enabling the replication of the formation and dissociation of a hybrid composed of the target nucleic acid in the sample and the probe, accumulating the irreversible change caused by the interaction of the reagents, and then detecting the accumulated change.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 is a graph showing the change with time of an ESR signal intensity ratio obtained in Example 1. In this drawing, □ indicates a case where a probe, a target DNA and fluorescein were contained in a reaction system and light irradiation was carried out; ■ indicates a case where the probe, the target DNA and fluorescein were contained in the reaction system and the light irradiation was not carried out; and ♦ indicates a case where the probe and fluorescein were contained in the reaction system and the light irradiation was carried out.

Fig. 2 is a graph showing-the change with time of an ESR signal line width obtained in Example 1 in which the probe, the target DNA and fluorescein were contained in the reaction system and the light irradiation was carried out.

Fig. 3 is a graph showing the change with time of an ESR signal intensity ratio obtained in Example 3. In this drawing, □ indicates a case where a probe having both bonded terminals, a target DNA and fluorescein were contained in a reaction system and light irradiation was carried out; ■ indicates a case where the probe having both bonded terminals, the target DNA and fluorescein were contained in the reaction system and the light irradiation was not carried out; and ♦ indicates a case where the probe having both bonded terminals and fluorescein were contained in the reaction system and the light irradiation was carried out.

DESCRIPTION OF THE PREFERRED EMBODIMENTS

A detection method of the present invention is based on a principle which is quite different from conventional techniques, and it comprises detecting a double helix structure itself which a hybrid has, and then amplifying the detected signal. That is, the present invention is substantially different from a conventional method such as a PCR method in which a step of amplifying the number of copies of a target nucleic acid and a step of detecting the amplified target nuclei acid are separately carried out. In the present invention, the detected signal of the double helix structure is amplified, and so the amplification and the detection are made in one system, and effective analysis can be achieved by a simpler device at a low cost.

Furthermore, the method of the present invention intends to detect the formation of the double helix structure in forming a hybrid, and according to the present invention, it is unnecessary to separate excessive probes from the hybrid of the probe and the target nucleic acid (B/F separation). In the present

invention, the formation of the desired nucleic acid hybrid can be detected by setting conditions for precisely detecting the double helix structure alone, even if a nonspecific adsorption or a mismatch is present, and thus the present invention can improve the precision of the measurement.

The present invention can be applied to the formation of the double helix structure such as DNA-DNA hybridization or DNA-RNA hybridization.

Now, the present invention will be described in detail.

A phenomenon called the hybridization has been heretofore only considered to be based on a hydrogen bond between the mutually complementary bases of a nucleic acid, because in general, after the immobilization of the nucleic acid (DNA or RNA), a hybridization reaction is carried out. However, in the case of the hybridization reaction in a solution, the formation of the double helix structure can be expected, if the nucleic acid forms double strands having a certain length. The present inventors have paid much attention to the fact that the nucleic acid having a single strand is different from the nucleic acid having the double strands (the hybrid) in a higher-order structure and chemical properties, and its detection system has been established. In consequence, the present invention has been completed.

In the double helix structure, the base portion of the nucleic acid forms a base pair by a hydrogen bond, and the helix of the nucleic acid is wound, a phosphorus portion and a saccharide portion being outwardly oriented. The nucleic acid bases are mutually stacked to be stabilized, and positioned at the center of a helix axis. As kinds of double helix structure, A, B, C and Z types and their variants are known. These structures are different not only in the base sequence but also in a pitch length, the symmetricalness of the helix, the width of a groove, the depth of the groove and the like under the influence of a kind of ion or a salt concentration used at the time of annealing, and even if the same base sequence is used, it is considered that the double helix structures vary with conditions to be used. In general, DNA takes the B type structure, and in this case, the pitch length is 33.8 Å and the number of the nucleic acid base pairs per pitch is the 10 bases.

The present invention is directed to a method for detecting the formation of a double helix structure by using reagents capable of being made a detectable change by the utilization of the double helix structure which the hybrid has, and then measuring the chemical change of the reagents.

As these reagents, there can be utilized 2 or more kinds of reagents by which an interaction is brought about through the double helix structure to generate a detectable irreversible change.

In the method of the present invention, the reagents are allowed to coexist with a reaction system of a sample and a probe, and a target nucleic acid contained in the sample and the probe are reacted under conditions under which formation and dissociation of the hybrid are repeated. Afterward, the resultantly accumulated (amplified) detectable signal is detected.

Next, the method of the present invention will be described in reference to a typical example in which the transfer of an electric charge is employed as the interaction.

For example, as the reagents, there can be used an electron donor and an electron acceptor which carry out the electric charge transfer through the double helix structure, and the electron donor and the electron acceptor can be bonded to both the ends of the probe, respectively. In this case, the electron donor and the electron acceptor must be combined so that at least one of them may give rise to the detectable change which does not disappear as a result of the interaction (the electric charge transfer).

When the two kinds of reagents (the electron donor and the electron acceptor) are reacted with the sample, the electric charge transfer takes place between the electron donor and the electron acceptor through the double helix structure of the hybrid of the probe and the target nucleic acid which hybrid has been formed in the case that the target nucleic acid is present in the sample. As a result of the electric charge transfer, in at least one of the electron donor and the electron acceptor, a detectable change occurs. Next, this reaction system is exposed to conditions for dissociating the formed hybrid, so that the target nucleic acid becomes a free state again. On the other hand, at this time, the detectable change by the interaction of the reagents which are bonded to the probe dissociated from the hybrid is held, because the detectable change is irreversible. Afterward, the reaction system in such a state is exposed to conditions for forming the hybrid again, and at this time, the target nucleic acid is reacted with the probe to which the electron donor and the electron acceptor which have not taken part in the interaction are bonded. As a result, the double helix structure is formed, and the interaction occurs between the electron donor and the electron acceptor through the thus formed double helix structure, so that at least one of the electron donor and the electron acceptor gives rise to the detectable change. Furthermore, the formation of the hybrid and its dissociation are repeatedly carried out, and at this time, the target nucleic acid catalytically functions to successively bring about the interaction of the reagents bonded to the probe. In consequence, the molecular number of the reagent holding the detectable change can be increased, and in other words, an analytical signal can be amplified.

As described above, the present invention intends to amplify the detection signal instead of the target nucleic acid, and therefore, even if the target nucleic acid is present in a trace amount, the analyst can be achieved with a good sensitivity. Moreover, since it is not necessary to amplify the target nucleic acid, various kinds of expensive reagents required to amplify the target nucleic acid are unnecessary. In addition, in contrast to a case using a PCR method in which the step of amplifying the target nucleic acid and the step of detecting the amplified target nucleic acid are separately carried out, the amplification and the detection can be done in one system, which can simplify the analytical step.

In order to repeat the formation and the dissociation of the hybrid in the method of the present invention, various techniques can be utilized. For example, temperature conditions by which the formation and the dissociation of the hybrid are equilibrated can be used to obtain the repeating reaction.

When the temperature conditions are used, it depends upon the length of the probe or the target nucleic acid, a salt concentration in a solution and so forth whether this equilibrium condition slants to a single-strand side or a double-strand side, and therefore the effective conditions should be suitably set. In general, the equilibrium state slants to the double-strand state at a low temperature (30 °C or less), and it slants to the single-strand state at a high temperature (70 °C). However, from the viewpoint of the good efficiency of the amplification, it is preferable to employ the temperature of a melting point of the nucleic acid at which the change of the nucleic acid between the single strand and the double strand is most rapidly carried out, a neighborhood of the melting point, or suitably a temperature in the range of ±5 °C of the melting point.

In measuring the melting point of the nucleic acid, an error is often unavoidable, and so there is a gap between the set melting point and the actual melting point sometimes. Furthermore, in the long nucleic acid, the higher-order structure of its base sequence gives rise to a difference between the measured and set melting point and the actual melting point sometimes. In order to avoid the disturbance of the amplification effect caused by such an error, or in order to positively and stably bring about the change between the single-strand state and the double-strand state, the temperature may be fluctuated within a certain temperature allowance of the melting point of the nucleic acid, suitably within a temperature allowance of ±5 °C of the melting point.

When the electric charge transfer is utilized as the interaction, as described above, the reagents are not limited to the electron donor and the electron acceptor, and at least a pair of the electron donor and the electron acceptor may be contained in the reaction system. The interaction between both of the electron donor and the electron acceptor is detected as the change of a chemical structure, the change of an electron state of the electron donor, the electron acceptor or a third substance which can interact with it, or the change of the signal attributed to the changed substance before and after the formation of the hybrid.

A relation between the electron donor and the electron acceptor which is referred to in the present invention is decided by a relation between energy states of both. Therefore, in the present invention, the substance generally defined as the electron donor or the electron acceptor is not used as thus defined, and substances which can become the electron donor and the electron acceptor are selectively used in combination of two or more kinds of reagents. For example, anthracene is utilized as the typical electron donor and its oxidation-reduction potential has been measured; and on the other hand, it is well known that characteristics of anthracene have also been inspected as the electron acceptor.

As the interaction of the electron donor and the electron acceptor, the so-called through space and through bond are considered. The former includes, for example, a case where the electron donor and the electron acceptor interact through the stacked base pair of the nucleic acid, and a case where the interaction is based on a proximity effect of the electron donor and the electron acceptor with the change toward the double helix structure. As the latter (the through bond), there can be considered the transfer of an electric charge through the bases, the phosphorus portion and the saccharide portion constituting the nucleic acid. In either case, no restriction is put on the conformation of the interaction, so long as the interaction of the electron donor and the electron acceptor is attributed to the formation of the double helix.

The interaction of the electron donor and the electron acceptor via the stacked bases of the nucleic acid can be achieved as follows. When the electron donor and the electron acceptor placed at a position where they react with the double helix structure are separated from each other so sufficiently that the interaction cannot be naturally carried out, an electron released from the electron donor is successively delivered from the base to the adjacent base through an electron cloud spread on the base pair of the nucleic acid, and it finally reaches the electron acceptor. Another mechanism can be considered in which the electron acceptor conversely draws an electron from the base pair of the nucleic acid and this behavior is carried out in succession, and finally the electron is taken from the electron donor. In short, a mediator in the electric charge transfer is the base pair of the nucleic acid.

On the contrary, the interaction based on the proximity effect of the electron donor and the electron acceptor can be carried out in a case where the electron donor and the electron acceptor get closer to each other to such a degree as to permit the interaction owing to the formation of the double-stand structure. For example, if both of the electron donor and the electron acceptor are bonded to the probe and this probe is in the state of the single strand, they do not interact, and if the probe is hybridized with the target nucleic acid to form the double helix structure and to thereby cause the electron donor and the electron acceptor to get closer to each other, the interaction takes place. Therefore, the formation of the double helix structure can be detected by the occurrence of the interaction.

Incidentally, when the achievement of the electric charge transfer is difficult between the electron donor and the electron acceptor via the double helix structure, a substance called a mediator or a sensitizer which can mediate the electric charge transfer therebetween may be interposed.

As described above, it is necessary that the electron donor and the electron acceptor are placed at the position where they react with the double helix structure to carry out the interaction therebetween. In order to place the reagents at the position where they react with the double helix structure, they may be inserted between the base pair of the nucleic acid as an intercalater, buried in the groove of the double helix structure, or disposed so as to snuggle to the double helix structure. In any case, it is essentially necessary for the present invention that they are specifically placed to the double helix structure of the hybrid formed from the single strand probe and the target nucleic acid.

Above all, the intercalater is most advantageous in the case that the electric charge transfer via the stacked base pairs is utilized. That is, the intercalater is usually a lamellar compound having a spread electron cloud, and it is arranged on an extended line of the stacked base pairs of the nucleic acid, at the same distance as the distance between the base pairs of the nucleic acid, and in parallel with the base pairs of the nucleic acid. For example, if the intercalater as the electron donor and the electron acceptor are arranged on the opposite sides of the double helix structure, the electron released from the electron donor is delivered from the base to the adjacent base through each electron cloud of the base pairs of the nucleic acid, so that the electron straight streams toward the electron acceptor. Alternatively, if the intercalater as the electron acceptor and the electron donor are arranged on the opposite sides of the double helix structure, an electron hole of the electron acceptor conversely draws an electron from the adjacent base pair of the nucleic acid and this electron drawing behaviors is carried out in succession between the other base pairs of the nucleic acid, and finally the electron is taken from the electron donor to terminate the electric charge transfer. In view of these mechanisms, in the case of the electric charge transfer via the stacked base pairs, it is preferable that at least one of the electron donor and the electron acceptor is the intercalater, and it is more preferable that both of them are the intercalators, because the efficiency of the electric charge transfer can be improved.

As a technique for detecting the change of the interaction of two or more kinds of reagents which is caused by the formation of the double helix structure, there is a method for detecting the change of the electron acceptor. This method can be classified into several categories in compliance with detecting means.

For example, the transferred electric charge can be observed as a spectrum change in accordance with a spin decoupling method using a spin labelling agent by ESR or the like. Alternatively, the transferred electric charge can be observed as the appearance or the change of a new absorption spectrum, as an electric charge transfer absorption band. In a system in which a solution is colored or discolored as a result of the electric charge transfer, the change can be directly observed with the naked eye, and it is further effective as the simple system. A luminescent system such as fluorescence or phosphorescence can also be utilized. In this case, there can be utilized a reaction by which fluorescence or phosphorescence is freshly generated, or a reaction in which the luminescence disappears as a result of the interaction. Alternatively, another method can be utilized in which the electron acceptor is chemically converted into another substance as a result of the electric charge transfer, and this converted substance is then detected. In this case, a third substance can be added to the converted substance to generate chemical luminescence by the chemical reaction between both the substances. When a protein such as an enzyme or an antibody is utilized as the third substance, a detection method using biological luminescence is utilizable.

The detection of the interaction may be achieved by detecting the change of the electron donor in addition to the change of the electron acceptor. Fundamentally, most of the methods for detecting the change of the electron acceptor can be directly applied. When a fluorescent substance is used as the electron donor, a direct change such as the disappear of fluorescence can be detected by the utilization of the fact that the quantum yield of the fluorescence is decreased by the electric charge transfer, or alternatively, the occurred change may be combined with some reactions so as to permit the detection with the naked eye.

In the present invention, the electron donor can be activated by light to release an electron and the electric charge transfer can be then started, and in addition to this manner, a third substance by which the electron donor may be stimulated to release the electron may be utilized.

Furthermore, in place of the electron donor, the electron acceptor may be activated to draw the electron from the electron donor. In this case, any initiator such as light may be used, as in the case of the electron donor.

As described above, a substance called a mediator or a sensitizer which can mediate the electric charge transfer may be interposed as the third substance in addition to the electron donor and the electron acceptor. This kind of substance interacts with the double helix to urge the electron donor or the electron acceptor not directly bonded to the double helix to do the electric charge transfer.

Needless to say, the non-bonded electron donor and electron acceptor which are free in a reaction system are also utilizable, so long as the interaction of both the free reagents specifically occurs only at a position where the double helix structure is present.

However, in a certain case, one or both of the electron donor and the electron acceptor are present in a free state in the reaction system, and the interaction occurs therebetween irrespective of presence/absence of the double helix structure, so that a background rises and an S/N ratio declines. In such a case, it is preferable that one or both of the electron donor and the electron acceptor are bonded to the probe, when used. If the rise of the background is inhibited by suitably selecting specific concentrations of these free reagents in the reaction system, the free reagents can be used at such concentrations. When the electron donor and/or the electron acceptor is bonded to the probe at the time of the utilization, the bonding of the electron donor and/or the electron acceptor to the probe is carried out via a linker such as $(CH_2)_n$, if necessary. In this case, the positional relation of the electron donor and the electron acceptor should be considered so that the interaction may be most effectively made.

The most suitable embodiment is a case where both of the electron donor and the electron acceptor are bonded to the probe as described above. In this case, the positional relation between the reagents for carrying out the interaction is definite, and therefore-the control of the interaction can be advantageously achieved by regulating the positional relation of these reagents with respect to the probe. In this case, a distance between the electron donor and the electron acceptor on the probe can be suitably selected in compliance with the kinds of these reagents. When a vicinal effect is utilized, for example, when the interaction is obtained by the vicinal effect, the distance between the electron donor and the electron acceptor is preferably in the range of from 20 to 120 Å, more preferably from 50 to 80 Å. Furthermore, when the electric charge transfer is carried out via a double helix structure, the distance is preferably in the range of from 20 to 120 Å, more preferably from 50 to 80 Å. The positions of the probe to which the electron donor and the electron acceptor are bonded depend upon the length of the probe, but it is advantageous from the viewpoint of the facility of the bonding that they are separately bonded to both the ends of the probe.

The length of the probe is suitably selected so that a good hybridization with the target nucleic acid may be possible and a stable double helix structure may be obtained. However, when both of the electron donor and the electron acceptor are bonded to the probe and they are close to each other, the interaction occurs in a certain case, even if the double helix structure is not present. Thus, the length of the probe is decided in consideration of such a case, but for example, it is a length of 8 or more bases, preferably a length of 12 or more bases.

However, in addition to the length of the probe, the base sequence itself as well as a salt concentration and an ion intensity in the reaction system have a large influence on the stabilization of the double helix structure. A G-C base pair contains more hydrogen bonds than an A-T base pair, and therefore, in the sequence containing many G-C base pairs, the more stable double helix structure can be formed. It is considered that if the molar concentration of KCl is raised from 0.01 M to 1 M, the melting point of DNA rises as much as 30 • C. In addition, the presence of the intercalator also largely contributes to the stability of the double helix structure. Therefore, the suitable utilization of these stabilizing factors permits the use of the probe having a length of less than the 8 bases.

Typical examples of the reagents which can be used in the present invention as follows:

Examples of the spin labelling agent include 4,4-dimethyloxazolidine-N-oxyl (DOXYL), its derivatives, 2,2,5,5-tetramethylpyrrolidine-N-oxyl (PROXYL) and its derivatives, and 2,2,6,6-tetramethylpiperidine-N-oxyl (TEMPO) and its derivatives.

Examples of the fluorescent intercalator include acridine, anthracene, pyrene, ethidium bromide, pyrylium, proflavine, porphyrin, thiazole orange dimer (TOTO), oxazole yellow (YOYO), and derivatives thereof.

Examples of the other general fluorescent dyestuff include cyanine, azulene, trinuclear dyestuffs, dansyl, fluorescein, Eosine, Rhodamine and riboflavin.

These compounds can be suitably used as the electron donor, the electron acceptor and the mediator for the sake of the evaluation of oxidation-reduction potential and the like.

The method of the present invention has been described in reference to the case where the electron donor and the electron acceptor for carrying out the electric charge transfer are used as the reagents, but these reagents are not limited to substances for carrying out the interaction by the electric charge transfer. Thus, the optional reagents can be utilized, so long as they can give rise to a detectable and irreversible change by the interaction via the double helix structure.

Now, the present invention will be described in detail in reference to examples, but the scope of the present invention should not be limited to these examples.

Example 1

[1] Preparation of a 20-mer oligonucleotide probe combined with a spin labelling agent TEMPO (4-hydroxy-2,2,6,6-tetramethylpiperidine)

(1) Synthesis of 4-aminohexylamino-2,2,6,6-tetramethylpiperidine-N-oxyl(4-aminohexylamino-TEMPO)

0.5 mmol of 4-oxo-TEMPO and 5 mmol of hexamethylenediamine dihydrochloride were dissolved in 30 ml of methanol, and 0.4 mmol of sodium cyanoborohydride and molecular sieves 3A were then added thereto. Afterward, the mixture was stirred at room temperature for 24 hours to react them. Next, the reaction solution was filtered through a glass filter to remove the molecular sieves, and the solvent was then removed from the filtrate under reduced pressure. To the thus obtained residue, 30 ml of 1-N hydrochloric acid was added, and it was then dissolved therein, followed by extraction with chloroform. The resulting chloroform phase was washed with water, and chloroform was then distilled off under reduced pressure. Afterward, water was added to the resulting residue, and insolubles were removed by filtration. The resulting filtrate was subjected to distillation under reduced pressure again to remove the solvent, thereby obtaining a reddish oily product.

(2) Synthesis of an oligonucleotide

20-mer oligonucleotide a base sequence of which is complementary to a portion of M13mp18DNA as a target DNA (a single strand) was synthesized with a 381A DNA automatic synthesizing device made by ABI Co., Ltd. In this case, a 5' terminal dimethoxytrityl group was removed on the automatic synthesizing device. Its base sequence was as follows:

5'-GTTGTAAAACGACGGCCAGT-3'

(3) Synthesis of a spin label oligonucleotide probe

The oligonucleotide (1 μmol) synthesized in the above-mentioned step (2) was transferred to a gas tight syringe, while bonded to a CPG support. The subsequent reactions were carried out in the syringe. Next, 1 ml of dioxane in which 50 mg of carbonyl-N,N'-diimidazol (CDI) was dissolved was added to the CPG support, and the solution was then allowed to stand at room temperature for 1 hour. After washing with dioxane and then drying under reduced pressure, a DMSO solution (0.4 ml of 0.2 M) of 4-aminohexylamino-TEMPO was added, and the solution was allowed to stand at 55 °C for 24 hours, washed with DMSO, dioxane and methanol in this order, and then dried under reduced pressure.

The spin label oligonucleotide was cut out and the protective was eliminated with concentrated aqueous ammonia in an ordinary manner, followed by purification with RPLC.

[2] Formation reaction of a hybrid of a TEMPO probe and M13 mp 18 DNA

0.2 μM of an oligonucleotide probe having TEMPO prepared in the above-mentioned [1] and M13 mp 18 DNA (0.2 pM, made by Takara Shuzo Co., Ltd.) were heated up to 80 °C in 1 mM phosphorus buffer solution (pH = 7.0)/145 mM NaCl/5 mM KCl, and the solution was then slowly cooled to a melting point (63 °C) of the hybrid, so that the probe and the target DNA were maintained in the equilibrium state of a single strand and a double strand. Next, fluorescein (made by Kodak Co., Ltd.) was added to this reaction solution so that a final concentration might be 10 μM, to prepare the same seven samples in all. These

samples were kept up under conditions for maintaining the equilibrium state of the single strand and the double strand, i.e., at a constant temperature of 63 °C, and they were irradiated with light of 490 nm for 0, 20, 40, 60, 80, 100 and 120 minutes by the following light irradiating device. Afterward, the samples were cooled to room temperature, and the measurement of ESR was then carried out.

Furthermore, the same procedure as described above was carried out except that M13 mp 18 DNA was not used, to prepare samples (probes alone) for the measurement of ESR spectrum, and the measurement of ESR was done in like manner.

[3] Measurement of ESR spectrum

The measurement of ESR spectrum in each of the samples was made by sweeping the sample every 20 minutes, and intensity ratio and line width were then measured. In the above-mentioned ESR measurement, a measuring device made by JEOL, Ltd. was used, and a flat cell made of artificial quarts was used.

The ESR measuring device and the light irradiating device were set as follows:

Table 1

Frequency	9.42 GHz
Modulation	100 kHz, 0.1 mT
Field	335 mT
Time Constant	0.3 sec
Power	10 mW
Sweep Time	8 min
Receiver Gain	1.25 x 1000

Light irradiating device	
Monochrometer	490 nm
Power supply	88.5 V-89 V/22 A

Fig. 1 shows a change with time of the ESR signal intensity in a case where a TEMPO-oligonucleotide probe alone was irradiated with light, a case where fluorescein/a TEMPO-oligonucleotide probe/M13 mp 18 DNA was irradiated with light, and a case where the light irradiation was not given. In addition, Fig. 2 shows a change with time of the line width of the ESR signal in the case that fluorescein/a TEMPO-oligonucleotide probe/M13 mp 18 DNA was irradiated with light.

As understood from Figs. 1 and 2, in the case of the TEMPO-oligonucleotide probe alone, the change of the intensity ratio was not observed, even when the light irradiation was carried out. Moreover, also in the case of fluorescein/the TEMPO-oligonucleotide probe/M13 mp 18 DNA, the change of the intensity ratio was not observed, when the light irradiation was not done.

On the contrary, in the case that fluorescein/the TEMPO-oligonucleotide probe/M13 mp 18 DNA was irradiated with light (490 nm), the intensity of ESR decreased with time (Fig. 1), and therefore the amount of TEMPO in which the spins were decoupled increased with time. Thus, it was confirmed that a detection signal was amplified. Furthermore, judging from the fact that any change of the line width was not observed (Fig. 2), it can be considered that the intensity change in ESR is not attributable to a chemical change. Accordingly, it can be understood that the spins of TEMPO were decoupled as a result of the transfer of the change from fluorescein to TEMPO via the probe/M13 DNA double strands, whereby the detection signal is amplified.

Example 2

The same experiment as in Example 1 was carried out except that the following sequence was used as the base sequence of a probe:

5'-GTTGTAAAAGGACGGCCAGT-3'

This sequence of the probe was that of the probe used in Example 1 in which the 10th base from the 5' terminal was changed from C to G, and therefore the probe sequence used in this example was different from that used in Example 1 in one base so as to mismatch with M13 mp 18 DNA.

To 0.2 μM of the probe having this mismatching sequence, 0.2 pM of M13 mp 18 DNA was added, and the solution was placed at a melting point by the same procedure as in Example 1. Next, fluorescein was added to the solution, and this solution was irradiated with light, and then cooled to room temperature. Afterward, changes with time of the intensity ratio and the line width of an ESR signal were inspected.

As a result, the intensity change seen in Example 1 was not observed, and the same intensity as in the case of the TEMPO-oligonucleotide probe alone was kept up. This fact indicates that a normal double-strand chain was not formed in a hybrid of the mismatching oligonucleotide probe and DNA, and an electric charge did not transfer, so that a signal was not amplified.

Example 3

Synthesis of a (5'-TEMPO, 3'-FITC) oligonucleotide probe labeled at both terminals

(1) Synthesis of an oligonucleotide combined with a 3'-amino group

20-mer oligonucleotide a base sequence of which is complementary to a portion of single-strand M13mp18DNA as a model of the target DNA used in Example 1 was synthesized with 381A, made by ABI Co., Ltd., using 3'-aminomodifier CPG (1 μmol) made by Gren Research Co., Ltd. as a carrier. A 5' terminal dimethoxytrityl group was removed on the automatic synthesizing device.

(2) Synthesis of a spin label oligonucleotide probe

An oligonucleotide (1 μmol) synthesized in the above-mentioned step (1) was transferred to a gas tight syringe, while bonded to a CPG support. The subsequent reactions were carried out in the syringe. Next, 1 ml of dioxane in which 50 mg of carbonyl-N,N'-diimidazol (CDI) was dissolved was added to the CPG support, and the solution was then allowed to stand at room temperature for 1 hour. After washing with dioxane and then drying under reduced pressure, a DMSO solution (0.4 ml of 0.2 M) of 4-aminohexylamino-TEMPO shown in Example 1 was added, and the solution was allowed to stand at 55 °C for 24 hours, washed with DMSO, dioxane and methanol in this order, and then dried under reduced pressure.

Next, the cut and deprotection of a spin label oligonucleotide were carried out with concentrated aqueous ammonia in an ordinary manner, followed by purification with RPLC, to obtain the spin label oligonucleotide in which TEMPO was labeled at a 5' terminal.

(3) Synthesis of an oligonucleotide probe labeled at both terminals

To the 3' terminal of a spin label oligonucleotide synthesized in the above-mentioned (2), a 3-amino-2-hydroxy group was bonded, and therefore the following operation was carried out to combine this amino group with FITC (fluorescein isothiocyanate, made by Sigma Co., Ltd.).

0.2 μmol (700 μl of aqueous solution) of the spin label oligonucleotide synthesized in the above-mentioned (2) was mixed with 100 μl of a 1 M sodium carbonate buffer solution (pH = 9), and a DMF solution containing 2 mg of FITC was added to the resulting solution and reaction was then carried out at 35 °C for 24 hours. Afterward, the reaction solution was treated by a gel filtration column NAP-25 made by Falmasia Co., Ltd. to remove a large excess of FITC, and then purified by RPLC to obtain a probe in which TEMPO was bonded to a 5' terminal and FITC was bonded to a 3' terminal.

(4) Measurement of ESR spectrum

0.2 μm of this probe and 0.2 pM of M13 mp 18 DNA were used, and the change with time of the intensity ratio and the line width of an ESR signal were inspected in the same manner as in Example 1. Between the probe alone and a hybrid of the light-unirradiated probe and a targe nucleic acid, any change of the intensity was not observed. On the contrary, only when the hybrid of the light-unirradiated probe and the targe nucleic acid was irradiated with light of 490 nm which was the excitation wave length of fluorescein, a signal was decreased with time, whereby the transfer of an electric charge could be confirmed (Fig. 2). Furthermore, the degree of spin decoupling was higher than in the case that fluorescein was not bonded to the probe, which meant that when the electron donor and the electron acceptor were bonded to both the terminals of the probe, the electron transfer took place more effectively, and in consequence, an effective signal amplification of the nucleic acid probe was attained.

Example 4

An oligonucleotide (the same sequence as in Example 2) having a base sequence which was different from that of M13 DNA in one base in the middle of a probe was synthesized, and the same operation as in Example 3 was carried out to bond TEMPO and fluorescein to both the terminals of the probe.

After 0.2 μM of this probe and 0.2 pM of M13 mp 18 DNA were used in a usual manner, the change with time of the intensity ratio and the line width of an ESR signal were inspected in the same manner as in Example 1. Even when a probe/DNA composite was irradiated with light of 490 nm which was the excitation wave length of fluorescein, changes of the intensity of the ESR signal and the line width were not observed, as in the cases of the probe alone and the light-unirradiated probe/DNA composite.

According to the present invention, the detection of a double-stand structure by a nucleic acid probe can be carried out by amplifying a detection signal in one system, whereby a target nucleic acid can be detected with high sensitivity by a very simple operation.

Furthermore, since the amplification is carried out by a purely chemical reaction without utilizing an enzyme as in a PCR method, problems such as the incorrect amplification or the retard of the amplification which are caused by an enzyme reaction can be avoided.

Particularly, in the case that the interaction of two or more substances is possible as a result of the transfer of an electric charge via the stacked bases of the nucleic acid, the proceed of the amplification is affected by the presence of one mismatch in a base sequence, and in consequence, it is possible to detect the one mismatch.

Furthermore, since the scatter of a temperature at the time of the amplification is not present or, if any, about 5 °C, an inexpensive device having a small capacity can be provided.

In addition, according to the present invention, the nucleic acid having 20 to 30 bases can be amplified in principle, and so the conventional problem that a short nucleic acid such as PCR cannot be amplified can be solved.

A method for detecting a target nucleic acid comprises the steps of reacting a sample with a probe in the presence of two or more kinds of reagents capable of being made an irreversible change capable of being detected and accumulating by an interaction through a double helix structure under a condition enabling the replication of the formation and dissociation of a hybrid composed of the target nucleic acid in the sample and the probe, accumulating the irreversible change caused by the interaction of the reagents, and then detecting the accumulated change.

Claims

1. A method for detecting a target nucleic acid which comprises the steps of reacting a sample with a probe in the presence of two or more kinds of reagents capable of being made an irreversible change capable of being detected and accumulating by an interaction through a double helix structure under a condition enabling the replication of the formation and dissociation of a hybrid composed of the target nucleic acid in the sample and the probe, accumulating the irreversible change caused by the interaction of the reagents, and then detecting the accumulated change.

2. The method according to claim 1 wherein said condition enabling the replication is a temperature condition of melting points of said target nucleic acid and said probe or vicinities of the melting points.

3. The method according to claim 1 or 2 wherein said interaction is based on a charge transfer between said reagents.

4. The method according to claim 3 wherein said two or more kinds of reagents include at least an electron donor and an electron acceptor.

5. The method according to claim 4 wherein charge transfer is carried out through stacked base pairs which are comprised in the double helix structure.

6. The method according to claim 4 wherein charge transfer is due to a proximity effect of said electron donor and said electron acceptor which is caused by the formation of the double helix structure.

7. The method according to claim 4 wherein said electron donor or said electron acceptor is a substance for bringing about a change in an EPR (electron spin resonance) spectrum by donating an electron or

accepting the electron, respectively.

8. The method according to claim 1 wherein said change based on the interaction between the reagents is optically detected.

9. The method according to claim 1 wherein said change based on the interaction between the reagents is chemically detected.

10. The method according to claim 1 or 4 wherein said reagents are intercalators to the bases of the nucleic acid comprised in the double helix structure.

11. The method according to claim 4 wherein said interaction between the reagents is started by light irradiation.

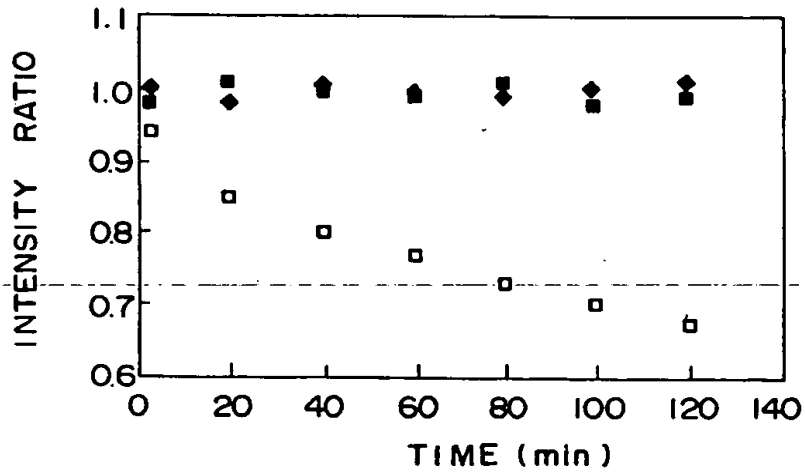
12. The method according to claim 1 or 4 wherein at least one of said reagents is bonded to the probe.

13. The method according to claim 3 wherein said change based on the interaction between the reagents is optically detected.

14. The method according to claim 3 wherein said change based on the interaction between the reagents is chemically detected.

15. The method according to claim 3 wherein said reagents are intercalators to the bases of the nucleic acid comprised in the double helix structure.

16. The method according to claim 3 wherein at least one of said reagents is bonded to the probe.


INTENSITY RATIO
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1.0
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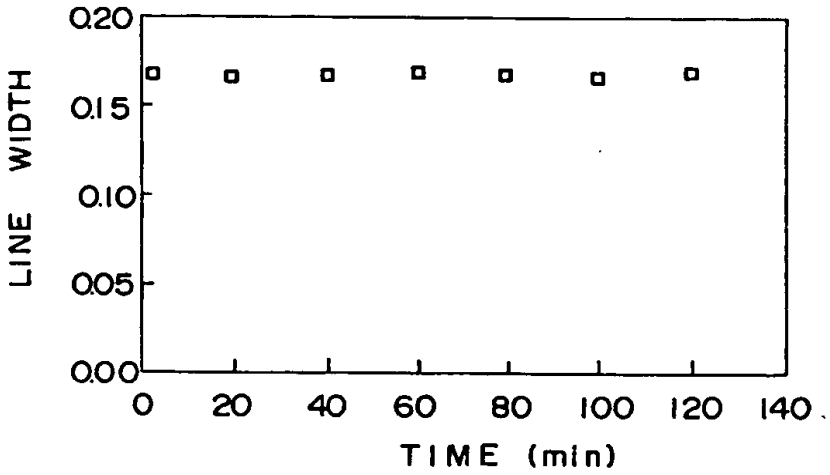
0.8
0.7
0.6
0
20
40
60
80
100
120
140
TIME (min)

FIG. 1


LINE WIDTH
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0.05
0.00
0
20
40
60
80
100
120
140
TIME (min)

FIG. 2

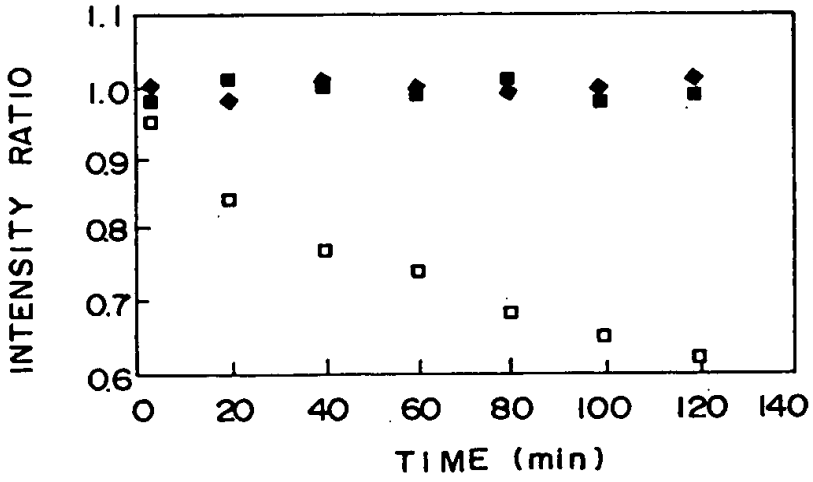
INTENSITY RATIO
1.1
1.0
0.9
0.8
0.7
0.6
0
20
40
60
80
100
120
140
TIME (min)

FIG. 3

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(54) **Method for detecting target nucleic acid.**

(57) A method for detecting a target nucleic acid comprises the steps of reacting a sample with a probe in the presence of two or more kinds of reagents capable of being made an irreversible change capable of being detected and accumulating by an interaction through a double helix structure under a condition enabling the replication of the formation and dissociation of a hybrid composed of the target nucleic acid in the sample and the probe, accumulating the irreversible change caused by the interaction of the reagents, and then detecting the accumulated change.

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EUROPEAN SEARCH REPORT

Application Number
EP 93 11 9104

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Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int.Cl.5)
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Y	NUCLEIC ACIDS SYMPOSIUM SERIES, vol. 27, 11 November 1992 LONDON GB, pages 97-98, T. SHIMIDZU ET AL. 'Syntheses of oligonucleotide derivatives with P(V)porphyrin and their properties' * the whole document *	1	
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C12Q

The present search report has been drawn up for all claims

Place of search	Date of completion of the search	Examiner
BERLIN	10 April 1995	De Kok, A

CATEGORY OF CITED DOCUMENTS

X : particularly relevant if taken alone
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European Patent Office

EUROPEAN SEARCH REPORT

Application Number
EP 93 11 9104

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Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int.Cl.5)
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The present search report has been drawn up for all claims

Place of search	Date of completion of the search	Examiner
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